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RE: Draft NTP Technical Report on SAN Trimer (TR 573), which is scheduled for review at the January 26, 2011 meeting of the NTP Technical Reports Peer Review Panel. See November 29, 2010, Federal Register notice (75 Fed. Reg. 73085).

Dear Dr White:

The Dow Chemical Company appreciates the opportunity to review the Draft National Toxicology Program (NTP) Technical Report on SAN Trimer (TR 573) (hereafter NTP technical report), that NTP released on December 8, 2010. The Dow Chemical Company (hereafter Dow) was founded in Midland, Michigan in 1897 and has grown into a diversified chemical company that harnesses the power of innovation, science and technology to constantly improve what is essential to human progress. Dow offers a broad range of products and services to customers in more than 175 countries, helping them to provide everything from fresh water, food and pharmaceuticals to paints, packaging and personal care products. Built on a commitment to its principles of sustainability, in 2009, Dow had annual sales of $45 billion and employed approximately 52,000 people worldwide.

Dow has a strong product stewardship program in place, which includes evaluation of potential risk from production, handling, and use of its products over their lifecycle. As a major producer of Styrene-Acrylonitrile polymers, which may contain the by-product SAN Trimer, Dow has conducted and participated in efforts to gather, summarize, evaluate, and supplement, available data to support development of risk assessments for these chemicals. Thus, Dow has a long history of providing and reviewing data to determine the potential hazards and risks of SAN Trimer and other related chemicals. Therefore, consistent with Dow's long-standing interest in

the use of sound science in determining safe uses for its products, we have participated in numerous scientific evaluations of SAN Trimer, and collaborative efforts on behalf of Union Carbide Corporation with the New Jersey Department of Public Health, the Environmental Protection Agency Region 2, and local groups, as an active member of the NTP work group focused on the review and evaluation of SAN Trimer.

Sound science is founded on a rigorously transparent and consistent process that includes critically evaluating the Draft NTP Technical Report on SAN Trimer (TR 573). The NTP technical report contains a number of errors and inaccuracies, lacks important details, and in some cases is lacking sections of important information. These issues should be addressed by the NTP and an updated technical report, in which the issues identified below have been corrected, should be made available to the NTP Technical Reports Peer Review Panel prior to the scheduled January 26, 2011 review meeting. Given the limited time to respond and the inability to conduct a full evaluation due to the unavailability of related study reports and missing information and details in the technical report, the comments offered below do not address all aspects of the technical report, but rather are directed towards several specific issues that are of most concern based on the information provided.

We find the NTP technical report conclusions on SAN Trimer are not supported by the evidence, specifically,

1. The cancer bioassay provides no evidence for carcinogenicity, but rather is consistent with background incidence for the CNS tumors and a determination of 'no evidence for carcinogenic activity'. Speculative statements about the links between certain effects and the tumors identified are not warranted, given that there were no tumors present at incidences above background levels.
2. There is no evidence for SAN Trimer-induced peripheral nerve degeneration; rather these observations are consistent with age-related neurodegeneration, a normal effect of aging in the rat identified at higher incidence due to the increased survival in the treated rats.

3. The weight of evidence indicates that SAN Trimer is not genotoxic. SAN Trimer is not mutagenic in bacterial or mammalian cells, and the DNA damage observed cannot be interpreted, or determined causal to any effects noted from SAN Trimer exposure, without provision of additional data and further analysis.
4. The bone marrow hyperplasia is a normal adaptive response to regenerative proliferation from hepatotoxicity.
5. Minimal to mild granulomatous inflammation observed in bone marrow at high doses in males and only mid-doses in females is a chance finding without biological significance.

The NTP technical report concluded that SAN Trimer presents "Equivocal Evidence of Carcinogenic Activity". This conclusion is based on the occurrence of astrocytomas and granular cell tumors in the brain and spinal cord. An "equivocal evidence" classification is assigned to "studies that are interpreted as showing a marginal increase of neoplasms that may be chemical related." The term "increase" implies that an elevation in the tumor incidence occurs above the concurrent control and/or historical background incidence for the specific tumor type. However, the incidence of brain tumors observed in all of the dosed groups was not significantly elevated above the concurrent controls and was within the historical control range. Specifically, the 4% incidence of astrocytomas found in male rats in the high dose group in the NTP study is within the historical control incidence of 0-4% for these tumors in male F344 rats (Sills *et al.*, 1999). In addition, the incidence of granular cell tumors (2%) observed in all SAN Trimer male rat groups in the NTP study is below the historical control rate (4%) for granular cell tumors of the brain in male F344 rats (Sills *et al.*, 1999). SAN Trimer did not produce a significant increase in astrocytoma or granular cell tumors compared with concurrent controls, nor an increase in these tumor types that was above the historical background incidence for these tumor types in the male F344 rat. Therefore, the evidence for carcinogenicity is not equivocal, but rather is nonexistent; there is no evidence for SAN Trimer carcinogenic activity in the male or female F344 rat. Thus the appropriate conclusion for SAN Trimer based on the draft NTP technical report is "No Evidence of Carcinogenic Activity".

According to the NTP technical report, spinal nerve root degeneration was observed in male rats and sciatic nerve fiber degeneration was observed in female rats that were attributed to chronic, lifetime, high-dose SAN Trimer exposure. Peripheral nerve degeneration occurs as a function of age in rats, with older animals exhibiting increased neurodegeneration compared with younger animals (Krinke, 1983). In the NTP study, there was improved survival associated with exposure to the test compound with the highest exposed rats living the longest (16% increase in survival of high dose males over controls). The NTP recognized the age-associated confounder and used a poly-3 statistical test in an attempt to control for the age-related confounding effects. The standard poly-3 test assumes that all animals had the same numbers of nerves examined. However, the standard poly-3 test may not be appropriate for evaluating nerve fiber degeneration in this study for several reasons, including that some animals may have had more spinal nerve roots and/or sciatic nerves examined than others. Unless the same number of nerves were scored for each animal, using the poly-3 test was not appropriate. NTP should make public the details for the statistical analysis of the peripheral nerve tissues, including the individual animal data used for the statistical analyses.

The NTP technical report also reports an increased severity of nerve fiber degeneration in male rats, which NTP claims is even more important than the increase in incidence. However, it is difficult to understand how this conclusion is reached given the extremely small difference in severity scores between control (1.1) and high dose-treated male rats (1.3). It is not clear from the report whether the histological examination of the nerve fibers was conducted in a blinded fashion and if not, then the results could be attributed to the non-blinded review and a reexamination of these slides could just as easily render no difference at all. The small increase in severity (from minimal to mild) in peripheral nerve degeneration reported by the NTP in the longer surviving, high-dose, SAN Trimer-treated rats compared to the concurrent controls is easily explained by the fact that the treated animals were older than the controls at terminal sacrifice. Given the recognized and expected age-related increase in this effect, this observation should not be considered a direct treatment-related effect. The analysis should adjust appropriately for the expected increase in peripheral nerve degeneration based on the demonstrated increased longevity in the high dose-treated rats.

The NTP technical report summarizes results from genetic toxicology studies conducted with the same batch of SAN Trimer used in the current toxicology and carcinogenicity bioassays. The results confirmed the lack of mutagenicity reported for previous bacterial and mammalian studies, but observed that SAN Trimer, administered once daily for 4 days by gavage to male and female F344/N juvenile rats, was associated with significantly increased levels of DNA damage in brain cells from the cerebrum and cerebellum, measured by the comet assay, and increased chromosomal damage in peripheral blood reticulocytes, measured by the micronucleus assay. The NTP technical report notes that the positive results seen with SAN Trimer in juvenile rats for the comet and micronucleus assays may be cause for concern and makes statements linking DNA damage with neurodegenerative lesions: "DNA damage has been implicated in the pathogenesis of many neurologic disorders (Martin, 2008) including cancer. It is possible that potential CNS toxicity seen in the current studies is associated with DNA damage in brain cells." (NTP technical report, page 94). The NTP technical report further discusses a putative relationship between DNA damage in brain cells and peripheral blood and tumor target tissues (page 50) and, more specifically, increases in micronuclei with "positive results in the *in vivo* peripheral blood rodent micronucleus assay have been shown to have a high predictability for rodent carcinogenicity (Witt *et al.*, 2000)." (NTP technical report, page 94), despite having no data that demonstrated an increase over background for any tumor type in SAN Trimer-exposed rats. The report discussion goes on to speculate that "The presence of tumors in SAN Trimer-exposed groups only in the brain and spinal cord in the current study may be due to lower rates of DNA repair in CNS tissue or greater sensitivity of these cells to SAN Trimer-induced damage. Lower rates of DNA repair might result in an accumulation of DNA damage leading to neurotoxicity (Fishel *et al.*, 2007)." (NTP technical report, page 94) and that "brain cells are especially sensitive to SAN Trimer-induced DNA damage" (NTP technical report, page 94), clearly attempting to implicate the reported increases in comet assay parameters as causal to the background incidence of brain tumors observed in the cancer bioassay. The original study reports of these genotoxicity assays have not been made available and key data were not included in the draft NTP technical report, so important details cannot be assessed. The genotoxicity studies as currently reported present several deficiencies in information and some significant concerns, *e.g.*,

questions on study design and interpretation of results, that need to be addressed before any conclusions about potential associations with other lesions, including the non-significant brain cancer incidence and the neurodegenerative lesions, would be scientifically appropriate.

The comet assay does not provide direct evidence of DNA mutations, a necessary precursor event for cancer, but rather provides evidence for DNA damage (mostly single strand breaks). As noted by NTP, "The *in vivo* Comet assay in liver cells is currently undergoing multilaboratory validation trials for inclusion in the international genotoxicity testing battery" (NTP technical report, page 50). In other words, the comet assay has not been validated for use as a standard genotoxicity test. There are numerous reasons why this is the case and many of the issues were summarized in comments on EPA Document: Framework for Determining a Mutagenic Mode of Action for Carcinogenicity submitted by Albertini and Walker (2007). The relevant section of their comments addressing the comet assay is excerpted below (pages 7-8):

> "The Comet assay in its most often used alkaline version...measures several kinds of DNA damage in single cells (Singh, 1988; Olive, 1991; Fairbairn *et al.*, 1995), but not mutations. This version incorporates alkaline denaturation for unwinding DNA double helices, thereby allowing free ends resulting from DNA single strand breaks (SSB) to migrate in a gel during electrophoresis. The alkaline version of the Comet assay detects primarily SSB in DNA but these may not be important genotoxic endpoints as they are usually rapidly and correctly repaired without leading to lethal or mutagenic effects (Collins *et al.*, 1997). In addition, many of these "single strand DNA breaks" do not really exist in the cells or *in vivo*. Rather, like other forms of alkaline treatments of double stranded DNA, the alkaline environment denatures the DNA, actually producing the strand breaks. Many of these *in vitro* induced strand breaks are actually the other forms of DNA damage detected by the alkaline Comet assay, such as alkali labile sites, abasic sites, and even DNA repair in progress. Because of the variety of DNA perturbations detected in this version of the assay, with most being in the form of SSB and alkali labile sites, their toxicological significance is uncertain, requiring correlation with other measures of genotoxicity for interpretation."

As noted above, the NTP has not to date provided the additional genotoxicity study reports, thus the following is a partial assessment of the comet assay based on the summary provided in the technical report. No information was provided on tissue cytotoxicity, which is known to increase the type of DNA damage measured by the comet assay. Thus the increased damage observed could simply be an artifact of the tissue cytotoxicity stemming from the high doses of SAN Trimer used, rather than a direct effect of exposure to SAN Trimer. For example, since each animal's brain cells are prepared separately, the increased DNA damage in some groups may be the result of more cell death in that tissue. The tissues came from the same rats used for the micronucleus assay, which demonstrated considerable (~60%) leukocyte cytotoxicity. This level of tissue cytotoxicity is quite high for a comet assay, where typically cytotoxicity does not exceed 10% and thus any 'positive' result could be an artifact of cell death. In fact, the top dose (~300 mkd) approached the LD50 for SAN Trimer (~500 mkd) and, when considered cumulatively (~1200 mkd over 4 consecutive days), exceeded the acutely lethal dosage for SAN Trimer; clearly these were very toxic doses that would produce at least some, and possibly significant levels of tissue cytotoxicity.

Also not provided were clinical observation data for the individual animals, and histopathology assessments of the tissues used for the comet assays, the results of which would help in interpreting the observations. Importantly, there was no DNA damage observed by NTP in the rats given 75 mkd, which is very close to the top dosage in the cancer bioassay (1,600 ppm ~80 mkd), indicating that if SAN Trimer produces genetic damage, it would not have done so in the cancer bioassay. One clear principle of a mutagenic mode of action is that the mutation effect must occur at a lower dose than the cancer induction effect (Dearfield and Moore, 2005), which appears not to be the case for this dataset.

Of more concern is the lack of details regarding the statistical analyses of the comet data. Based on the minimal information provided in the summary, the NTP used some very novel approaches in its assessment of the comet data. The NTP counted 800 cells/tissue/rat, whereas the literature and standard guidance recommends 100-150 cells/tissue/animal (Wiklund and Agurell, 2003;

Lovell and Omori, 2008). The 4- to 5-fold increase above the recommended number of cells to count excessively increases the statistical power, most likely resulting in Type I error (false positive). Given the tremendously increased power to identify statistically significant changes, there was only a ~20% increase in DNA damage at the top dose, a very small increase considering the high concentration used in the assay (exceeding the LD50 for SAN Trimer by several-fold, over 4 days of dosing) and the large number of cells analyzed. No rationale was provided for the novel statistical analyses, which did not apply standard guidance. It is not clear whether this was an *a priori* approach included in a statistical protocol, or if the number of cells counted was increased until a significant difference was attained. NTP's departure from guidance recommendations also negates the ability to compare these results with appropriate historical control data, which is important given the very small differences observed in animals exposed to these extremely high doses of SAN Trimer.

Some additional observations for which no explanations were provided include that the positive control response (ethyl methanesulfonate) was not very robust, thus the NTP should provide the historical positive control data to understand typical dynamic range for the laboratory where the assay was conducted. Also, there is an unnoted and unexplained approximately two-fold difference between sexes in values for all doses (including vehicle controls) for liver and leukocytes for the comet study; it is not clear why this occurred, nor whether this is typical, or what, if any, are the implications of these apparently sex-related differences, especially as there were no brain tumors observed in female rats.

As noted for the comet assay, the micronucleus assay also does not provide direct evidence of DNA mutations, a necessary precursor event for cancer, but rather provides evidence for DNA damage as clastogenicity. Again the NTP provided only a summary of the assay and its results but not the study report, so a complete and thorough assessment is not possible here. Rather striking was the excessive toxicity observed in the positive control for the micronucleus assay, with no explanation for that observation or its implication for the validity of the assay. Of immense importance for any *in vivo* micronucleus assay is the clinical observation data and most importantly the body temperature data for the individual animals. Both hypothermia and

hyperthermia are recognized to induce small increases in micronuclei that could easily explain the small changes reported in this study. In addition, historical control data were not provided so it's not possible to determine if the small changes reported were within the historical control range for this testing facility. In other words, the small changes may simply be within the normal variation and not related to SAN Trimer exposure. Unfortunately, the data were not provided for such an assessment nor were the details for the statistical analysis included.

The NTP technical report noted incidences of bone marrow hyperplasia were significantly increased in 1,600 ppm males and females and 800 ppm females. However, the severity grade for the hyperplasia was greatest in the control animals over the high-dose animals, which had the highest number of animals with hyperplasia. NTP provides no explanation for the decrease in severity of bone marrow hyperplasia that was associated with SAN Trimer. Incidences of bone marrow granulomatous inflammation were increased in 1,600 ppm males, and 800 and 1,600 ppm females, and the increase in the 800 ppm females was significant. According to NTP this lesion is very rare and therefore should be considered biologically significant. However, the report does not elucidate what the biological significance might be. Moreover, the non-statistically significant increases of granulomatous inflammation at the high dose in 3 male rats and 2 female rats were scored as mild in males and minimal to mild in females. The significant increase in granulomatous inflammation in the mid-dose female rats was slightly above minimal. Moreover, there is a lack of a monotonic dose-response in the females, with no statistically significant effect at the low or high doses; the highest incidence and sole statistically significant effect was at the mid-dose. Therefore, the statistical significance at $P \leq 0.05$ appears to be a chance finding rather than a real effect with biological importance. It is worth noting that SAN Trimer reduced the incidence of leukemia at all doses with a monotonic dose-response.

NTP also reported hepatotoxicity with the incidence of eosinophilic foci and mixed cell foci significantly increased in the livers of male and female rats. Also observed was an increase in necrosis and inflammation. Thus, the bone marrow hyperplasia discussed above would be expected as an adaptive response to the hepatic toxicity with regenerative proliferation-induced stimulation of hematopoietic stem cell proliferation to replace the loss of hepatic cells.

Throughout the technical report, the NTP discusses the toxicity of acrylonitrile even though acrylonitrile was not tested in the NTP studies, nor was it quantifiably present in the test material, based on thorough analytical characterization.

> "Results of GC/MS and HPLC/MS analyses supported the composition of the bulk chemical as a mixture of trimers of styrene and acrylonitrile, and the results of the HPLC/MS analyses were consistent with data from the manufacturer's analyses of Batch 3. Subsequent analysis of the SAN Trimer for the presence of ... acrylonitrile monomers indicated that ...Acrylonitrile was not present above the limit of detection (0.008%)." (NTP technical report, page 30)

Clearly any SAN Trimer-associated effects discussed above in the NTP technical report, if treatment-related, were from SAN Trimer exposure alone and not due to acrylonitrile. The oft mentioned and sometimes lengthy references to acrylonitrile by the NTP only serve to confuse the points raised by the NTP, and provide no support or clarity to the scientific discussion. Therefore, NTP should remove the references to acrylonitrile throughout the technical report except perhaps in its description addressing the origin of the SAN Trimer.

As NTP further reviews the results of its studies, we encourage NTP to consider the issues raised in these comments. The cancer bioassay provides no evidence for carcinogenicity, but rather is consistent with background incidence for the CNS tumors. There is no evidence for SAN Trimer-induced peripheral nerve degeneration, rather these observations are consistent with age-related neurodegeneration, a normal effect of aging in the rat and present with increased incidence in the high-dose animals because of their increased age due to their longer survival. The weight of evidence indicates that SAN Trimer is not genotoxic; namely, SAN Trimer is not mutagenic in bacterial or mammalian cells, and the DNA damage observed cannot be determined to be caused by SAN Trimer without further analysis. The bone marrow hyperplasia is a normal adaptive response to regenerative proliferation from hepatotoxicity and the minimal-to-mild

granulomatous inflammation observed in bone marrow at high doses in males and only mid-doses in females is a chance finding without biological significance.

Thank you for your review and consideration of these comments. If you have any questions regarding these comments, please contact me at (989) 636-9063.

[Redacted]

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References:

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